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SEQUENTIAL SACCHARIFICATION AND SIMULTANEOUS FERMENTATION OF PINEAPPLE WASTE FOR BIOETHANOL PRODUCTION

Nur Hidayah Binti Hussin

(37967)

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NUR HIDAYAH BINTI HUSSIN

37967

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APPROVAL SHEET

Name of Candidate : Nur Hidayah binti Hussin

Title of Thesis : Sequential Saccharification and Simultaneous Fermentation of Pineapple Waste for Bioethanol Production

____

(Dr Micky Vincent)
Supervisor
Department of Molecular Biology
Faculty of Resource Science and Technology
Date:

____

(Dr Ho Wei Seng)
Coordinator
Department of Molecular Biology
Faculty of Resource Science and Technology
Date:

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DECLARATION

I declare that no portion of this research work has been submitted to support the application of other degree or qualification at any other universities or institution of higher learning.

(Nur Hidayah binti Hussin)
Resource Biotechnology Programme
Faculty of Resource Science and Technology
University Malaysia Sarawak
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LIST OF ABBREVIATIONS

ABS	Absorbance
ADF	Acid Detergent Fibre
ATCC	American Type Culture Collection
FPU	Filter Paper Unit
g	gram
HPLC	High Performance Liquid Chromatography
M	Mole
ml	millilitre
NaOH	Sodium hydroxide
NDF	Neutral Detergent Fibre
S. cerevisiae	*Saccharomyces cerevisiae*
SSSF	Sequential Saccharification and Simultaneous Fermentation
YMB	Yeast Malt Broth

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Sequential Saccharification and Simultaneous Fermentation (SSSF) of Pineapple Waste for Bioethanol Production

Nur Hidayah binti Hussin

Resources Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
94300 Kota Samarahan
Sarawak

ABSTRACT

Bioethanol is a renewable source of energy that can be used as an alternative to overcome the depletion of fuel for transportation. It is also friendly to the environment as the burning of bioethanol emits zero net carbon output to the atmosphere. In this study, pineapple wastes were used to produce bioethanol via Sequential Saccharification and Simultaneous Fermentation (SSSF) a modified version of Simultaneous Saccharification and Fermentation (SSF) by using cellulase and *Saccharomyces cerevisiae*. SSSF was performed on the pineapple waste at 5.0% (w/v) substrate load, with and without the presence of cellulase, for five days under anaerobic conditions to compare the bioethanol yield. Phenol-Sulphuric Total Carbohydrate assay and High Performance Liquid Chromatography (HPLC) were used to analyze the samples taken from fermentation broth. Based on the results, SSSF of 5% (w/v) treated pineapple waste gives higher ethanol yield compared to SSSF of 5% (w/v) untreated pineapple wastes which are 5.85 g/l and 2.54 g/l respectively at 120 hour of fermentation. This study has proven that SSSF of pineapple waste was able to liberate bioethanol and it can be considered to be used as a substrate for industrial scale bioethanol production in future.

Keywords: Sequential saccharification and simultaneous fermentation (SSSF), pineapple waste, cellulase and bioethanol

ABSTRAK

Bioetanol merupakan sumber tenaga yang boleh diperbaharui dan boleh digunakan sebagai alternatif untuk mengatasi masalah pengurangan bahan api untuk pengangkutan. Ia juga merupakan bahan yang mesra alam sekitar, kerana pembakaran bioethanol secara puratanya mengahasil kan sifar pengeluaran karbon ke atmosphera. Melalui projek ini, sisa buangan nanas digunakan sebagai bahan untuk menghasilkan bioetanol melalui proses sakarifikasi dan fermentasi serentak berperingkat (SSSF) dengan menggunakan Saccharomyces cerevisiae dan enzim selulase. SSSF dilakukan ke atas sisa buangan nanas pada 5.0 % (w/v) dengan kehadiran dan dengan ketidak hadiran selulase enzim selama lima hari tanpa kehadiran oksigen bagi membandingkan penghasilan bioethanol. S. cerevisiae merupkan mikroorganisma yang dipilih untuk melakukan proses fermentasi ke atas gula ketika proses fermentasi berjalan. Terdapat dua kaedah analisis utama yang digunakan bagi menganalis sampel fermentasi iaitu kromatografi cecair prestasi tinggi (HPLC) dan pengujian total karbohidrat phenol-sulfurik. Berdasarkan keputusan yang diperoleh,SSSF ke atas 5% (w/v) dengan enzim selulase menghasilkan etanol dalam jumlah yanglebih tinggi berbanding SSSF ke atas 5% (w/v) tanpa enzim iaitu 5.85 g/l dan 2.54 g/l pada masa ke 120 jam fermentasi. Kajian ini membuktikan bahawa SSSF ke atas sisa buangan nanas mampu menghasilkan etanol dan ia boleh digunakan sebagai substrat bagi penghasilan ethanol dalam skala industri pada masa hadapan.

Kata kunci: Sakarifikasi dan fermentasi serentak berperingkat (SSSF), sisa buangan nanas, selulase dan bioethanol.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Crude oil is known to be the world's top energy source, and, over 80% of the oil produced is used as a main energy source to accommodate the transportation sector (Graul, 2007). These types of fuels are non renewable sources of energy. According to Graul (2007), 30 million barrels of oil are needed daily to supply the needs to move cars and trucks, trains, ships and aircrafts all over the world. Apart from transportation, plastic products such as food container, plastic bags, toiletries, and pharmaceutical and some clothing materials are also made up from petrochemical (National Renewable Energy Laboratory, 2002). These applications are gradually depleting the fuel sources. Furthermore, the demands for fuel are increasing due to the growing number of the world population and the industrialization sectors (Sarkar et al., 2012). This can lead to world energy crisis if no alternative sources of energy are found to solve this problem.

Recently, bioethanol is widely used to substitute the usage of gasoline for transportation purposes. Brazil and United States are the world's two largest producers and consumers of bioethanol (Ong, 2008). As a renewable source of energy, bioethanol can be considered as a good alternative for fuel consumption apart from its clean and eco-friendly properties towards the environment (Bries, 2008). Apart from solving the depletion of crude oil problem, bioethanol consumption can also help to reduce the emission of greenhouse gases to the environment thus led to the environmental sustainability.

Pineapple is one of the crops that is being planted in Malaysia in a large scale. The state of Johor contributes to approximately 57% of the overall production of pineapple in Malaysia with 163, 830 metric ton pineapples produced in 2010 (Malaysia Pineapple Industry Board, 2012). Other states that produce pineapple in Malaysia include Selangor, Pulau Pinang and Kelantan (Malaysia Pineapple Industry Board, 2012). Basically, pineapples are subjected to the food canned industry sector. However, the by-product from this industry can contribute to environmental pollution, if improperly managed. Thus, by using pineapple waste as the substrate for bioethanol production, this can help to reduce the problem. Furthermore, pineapple waste can be obtained easily throughout the year. Pineapple waste consists of lignocellulosic materials that comprise of cellulose, hemicelluloses, lignin, sugar and other carbohydrates that can be fermented to produce bioethanol (Choonut et al., 2014).

In this study Sequential Saccharification and Simultaneous Fermentation (SSSF) was used to obtain the targeted product which is bioethanol. This is a modified version of Simultaneous Saccharification and Fermentation method. This method was very suitable to be used for the substrate that consist significant amount of sugar that can be directly converted into ethanol. After that, the appropriate enzyme was added to allow the hydrolysis of lignocellulosic materials into fermentable sugars which than can be fermented into ethanol. Thus, this help to optimize the efficiency of the enzyme used and consequently produced higher ethanol yield.

There are three stages involved in the production of ethanol. The first stage involves size reduction and pre-treatment of the substrate for delignification for the releasing of hemicelluloses and cellulose. The second stage is the hydrolysis of hemicelluloses and cellulose to produce glucose, xylose, arabinose, galactose, and mannose, and, lastly is the production of ethanol from the fermentation of sugar (Srimachai et al., 2014).

The aim of this project is to study the production of bioethanol from the pineapple waste via Sequential Saccarification and Simultaneous Fermentation (SSSF) using cellulase and *Saccharomyces cerevisiae* to further utilize pineapple canned industrial waste, as well as, producing bioethanol.

1.2 The objectives of this study are as follow:

1. To characterize the composition of the pineapple waste collected from the Kuching-Kota Samarahan area.
2. To produce and determine the efficiency of bioethanol production from pineapple waste via Sequential Saccharification and Simultaneous Fermentation (SSSF) using *S. cerevisiae.*
3. To determine the biomass reduction after SSSF.

CHAPTER 2

LITERATURE REVIEW

2.1 Bioethanol

Bioethanol is also known as ethyl alcohol, grain alcohol or ETOH with the chemical formula CH_3-CH_2-OH. Basically this liquid biofuel can be produced from several types of biomass feedstocks or wastes through conversion technologies (Balat et al., 2008). Ethanol belongs to the alcohol family, a group of organic chemical compounds that contain a hydroxyl group, (-OH), which is bonded to a carbon atom from the ethyl group (Shakhashiri, 2009). Ethanol is a clear, colorless, volatile and flammable liquid with a strong and distinctive odor (Shakhashiri, 2009). Bioethanol is an attractive alternative fuel as it is a renewable bio-based resource of energy and as it burns more cleanly thus it reduces the overall emission of carbon dioxide (Bawa, 2008). Thus it contributes to the reduction of environmental pollution.

Bioethanol has a higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization compared to conventional gasoline (Balat, 2008). These properties lead to the theoretical efficiency of bioethnol over gasoline in an internal combustion engine as it has shorter burn time and leaner burn engine (Balat, 2007).

The first generation of bioethanol is produced either from sugar-based ethanol plants that are predominantly produced in Brazil from sugarcane, or starch-based ethanol plant like corn and grains and is dominated by United States (US) followed by other major ethanol producing countries such as China, Canada, France, Germany, and Sweden (Lennartsson et al., 2014). As for the second generation of bioethanol, it is produced from the utilization of different types of lignocellulosic materials such as non-food materials and the waste parts available from the plants (Naik et al., 2010).

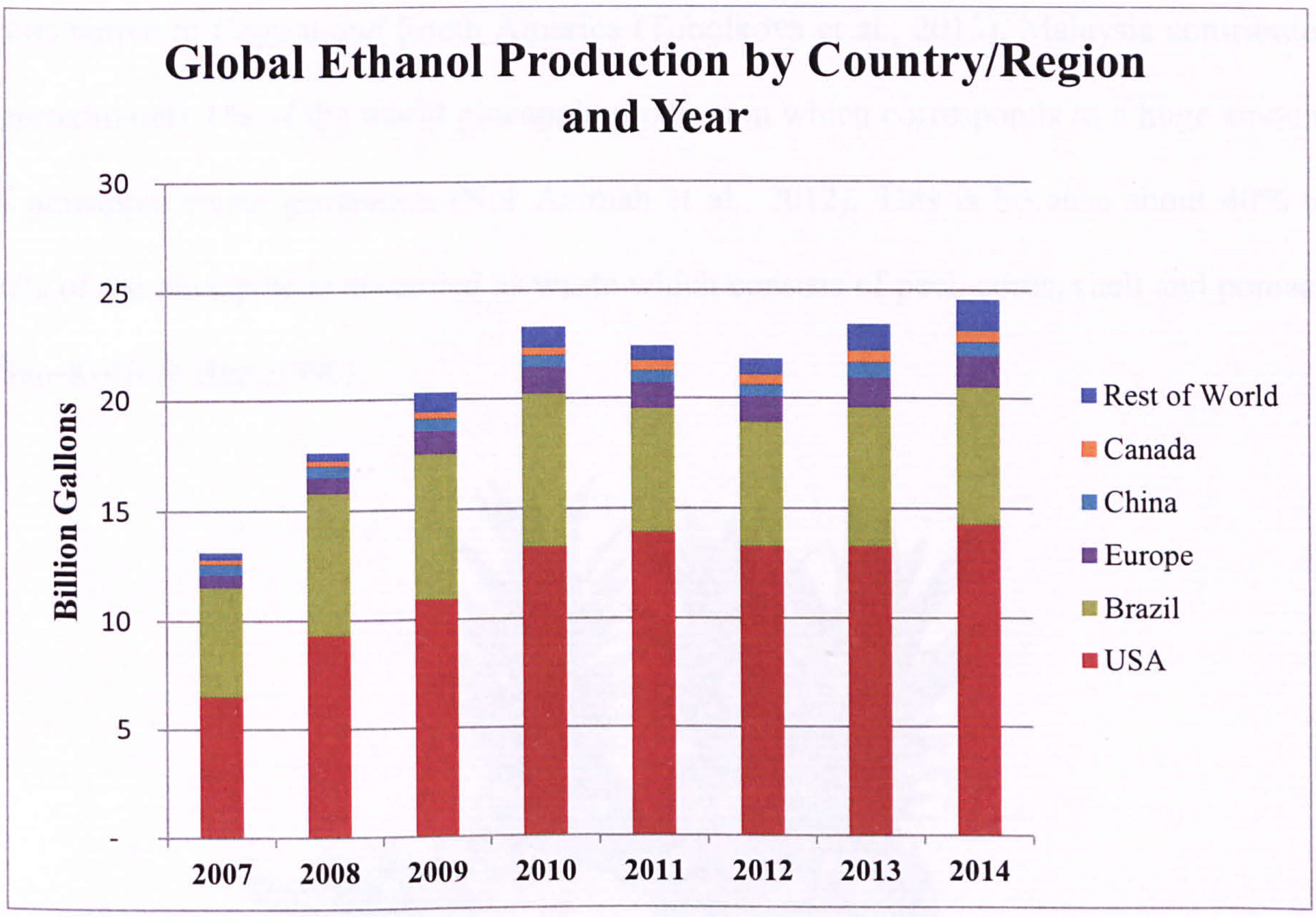

Figure 1: Annual production of bioethanol in selected country (www.afdc.energy.gov/data/).

2.2 Pineapple Waste

Pineapple or *Ananas cosmosus* as shown in Figure 2 belongs to tropical and sub-tropical fruits native to Central and South America (Tobolkova et al., 2013). Malaysia contributes approximately 1% of the world pineapple production which corresponds to a huge amount of pineapple waste generation (Nor Azimah et al., 2012). This is because about 40% to 80% of the pineapple is discarded as waste which consists of peel, cores, shell and pomace (Ban-Koffi & Han, 1990).

Figure 2: Pineapple fruits.

According to Koshy (1990), all parts of pineapple waste including the peeled skin, unwanted fruits and the core part will be sent for crushing process and then the solid waste will be sent to cattle feeding while the liquid waste will be stored for fermentation process. Figure 3 shows the pineapple canning process that basically takes place in pineapple canning industry.

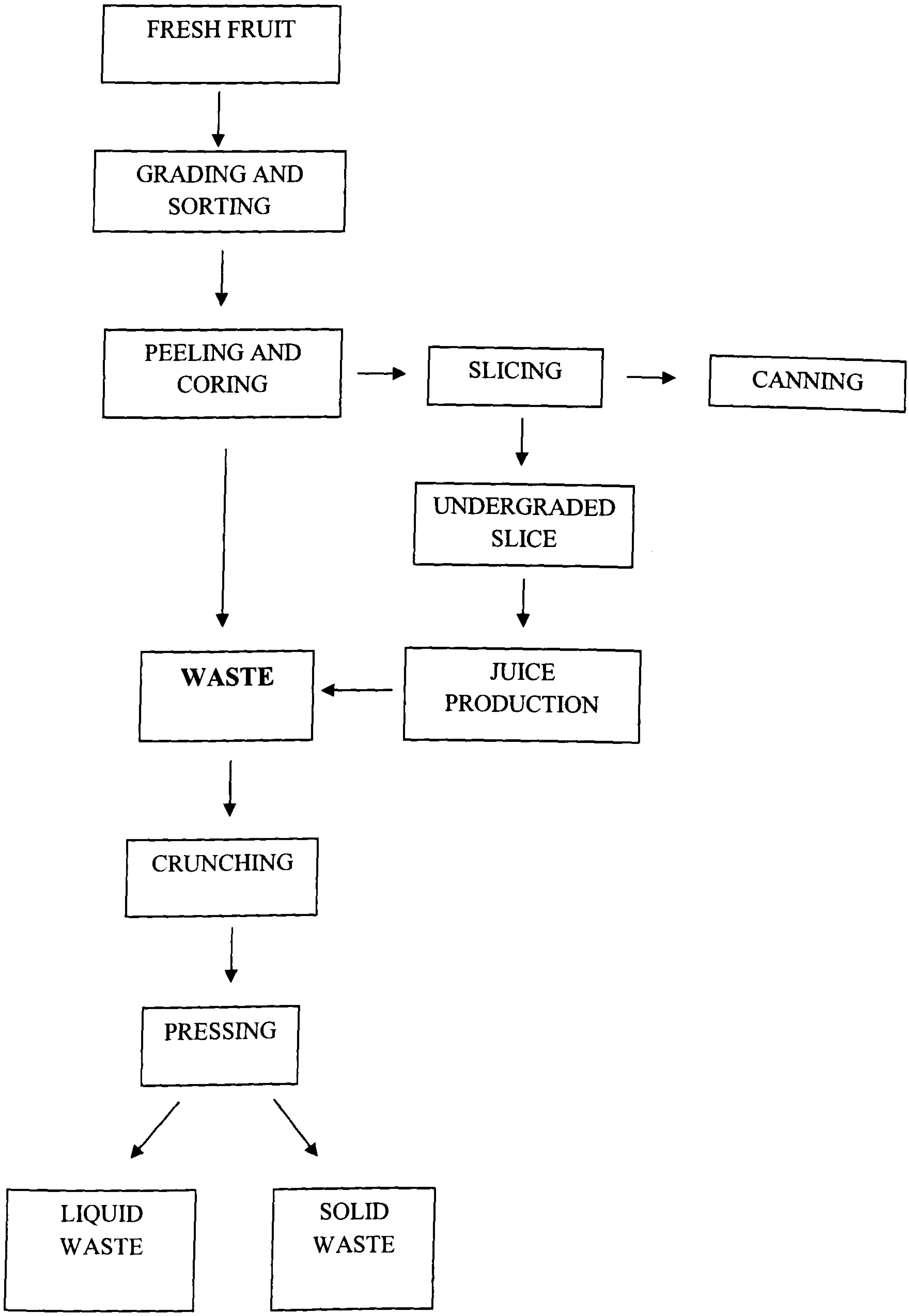

Figure 3: Pineapple canning process (Jackson & Shinnery, 1979).

Different parts of pineapple waste as shown in Figure 4, contain different amounts of soluble sugar and lignocellulosic materials depending on the geographical origins and varying degrees of ripeness (Hanapi & Abdullah, 2007). Pineapple waste contain high amount of reducing sugar compare to pineapple pulp (Hemalatha & Anbunselvi, 2013).

Figure 4: Different parts of pineapple waste.

However, pineapple waste contains relatively low amount of soluble sugar which included 5.2% sucrose, 3.1% glucose and 3.4% fructose. Whereas the lignocellulosic component present in relatively high amount including 19.0% cellulose, 22.0% hemicelluloses, 5.0% lignin and 53.0% cell soluble matter (Ban-Koffi & Han, 1990). Pineapple waste having a high level of biochemical oxygen demand (BOD) as well as chemical oxygen demand (COD), thus if the waste is not properly dispose, it can cause a serious environmental pollution problem (Burbank & Kumagai, 1965).

2.3 Lignocellulosic Biomass

Lignocellulosic materials comprise of two main classes of structural polysaccharides, which are cellulose and hemicelluloses (Tropea et al., 2013). Cellulose has linear and crystalline structure with a homopolymer of repeating sugar units of glucose linked by β-1,4-glycocidic bond, as shown in Figure 5 (Sarkar et al., 2012). Hemicellulose as shown in Figure 6, is a short and highly branched polymer with a heteropolymer of D-xylose, D-arabinose, D-glucose, D-galactose and D-mannose (Sarkar et al., 2012).

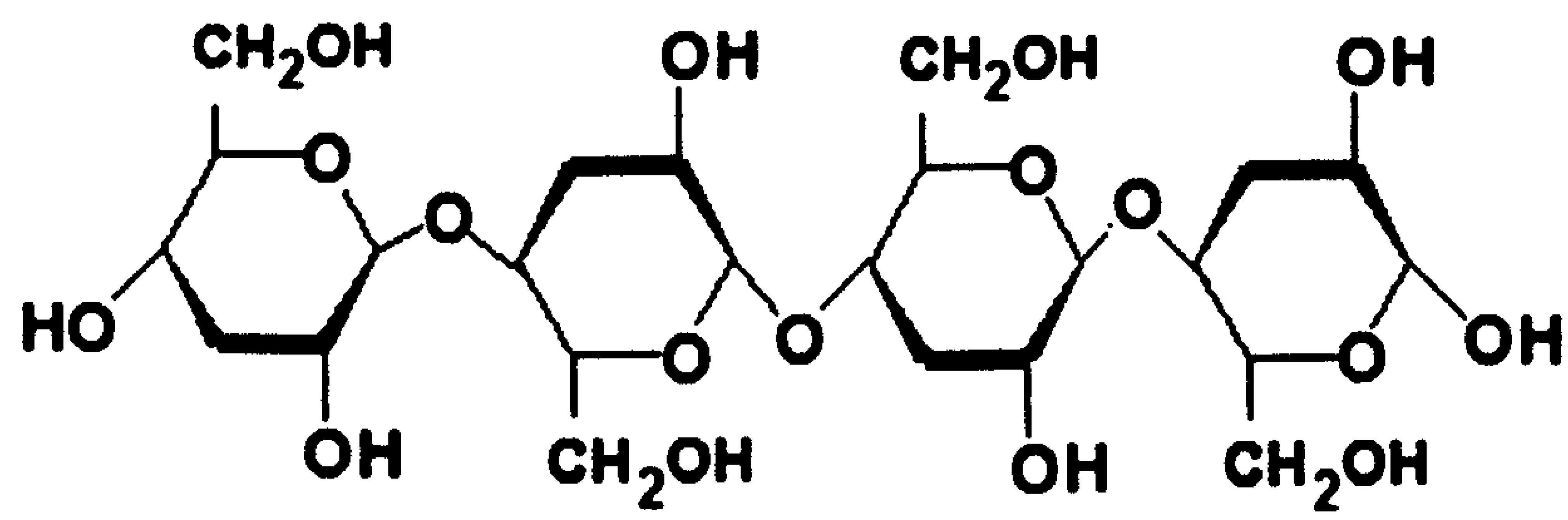

Figure 5: Structure of cellulose.

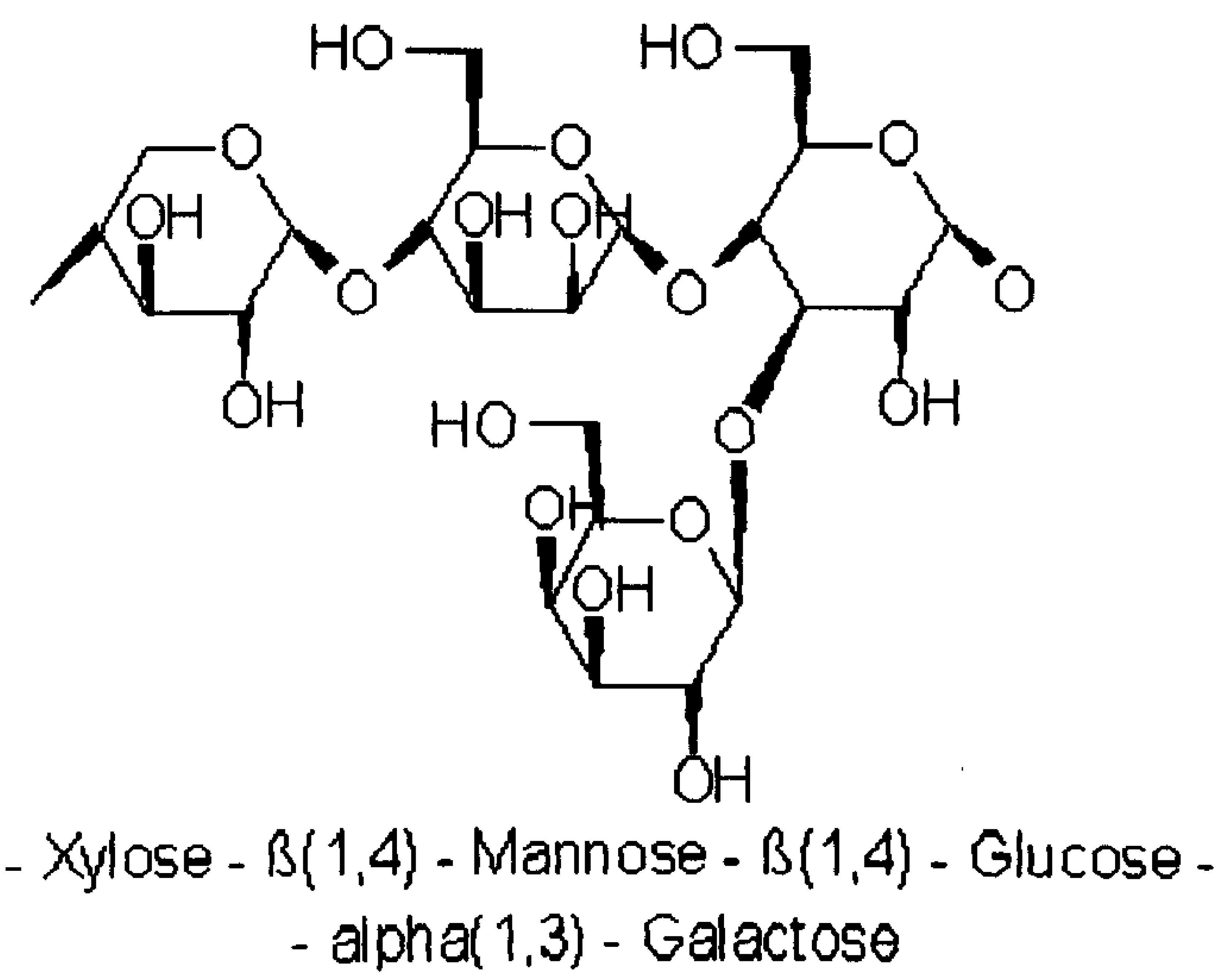

Figure 6: Structure of hemicellulose.

Hydrolysis of cellulose and hemicelluloses materials either by using enzymatic or chemical hydrolysis, produce fermentable sugars such as glucose, mannose, arabinose and xylose (Tropea et al., 2014). These sugars that are released from the hydrolysis reaction can then be fermented into ethanol by using *Saccharomyces cerevisiae.* The polysaccharides are tightly packed in plant cell walls and often surrounded by lignin, forming highly recalcitrant structures resistance to direct enzymatic attack (Sun & Cheng, 2002; Himmel et al., 2007). Lignin is hydrophobic in nature and will function as glue like materials to hold in place all the polysaccharides together as well as to protect cellulose and hemicelluloses from microbial attack (Peiji et al., 1997). After hydrolysis of the lignocellulose polysaccharides, lignin remains as a solid residue, although a minor part is degraded to phenolic and other aromatic compounds (Jonsson et al., 2013).

Lignocelullosic material become one of the most chosen substrate for fermentation, because it can be found in an abundant and can be obtain easily. According to Saratale and Oh (2012), the most abundant lignocellulosic agriculture by-product are corn cobs, corn stover, wheat rice, barley straw, sorghum stalks, coconut husks, sugarcane bagasse, switch grass, pineapple and banana leaves.

2.4 Cellulase

Enzymatic hydrolysis must be carried out in order to convert the lignocellulosic materials into fermentable sugars. Hydrolysis of cellulose requires the cocktail of a few enzymes collectively known as cellulase (Khanal et al., 2010). Cellulases are the enzymes that hydrolyze β-1,4 linkages in cellulose chain and usually they are produced by fungi, bacteria, protozoans, plant and animals (Zhang and Zhang, 2013). The main function of cellulase enzyme is to hydrolyze cellulose to D-glucose, which can then be fermented into alcohol in the presence of yeast (Krishna et al., 2001).

Basically, cellulase consists of a mixture of three different enzymes which are endo-β-1,4-glucanases, exo-β-1,4-glucanases (cellobiohydrolase), and β-1,4-glucosidases (Khanal et al., 2010). Endoglucanases, is a group of enzyme that will attack the middle chain of cellulose and cut β-1,4-bonds randomly on the cellulose chains, generating new ends (Zhang and Zhang, 2013). Most of catalytic modules of endoglucanases have a cleft/grove-shape active site which allows endoglucanases to bind and cleave the cellulose chain to produce glucose, soluble cellodextrins or insoluble cellulose fragment (Zhang and Zhang, 2013). For exoglucanases, the enzymes will act at the end of the cellulose chain (Khanal et al., 2010). According to Zhang and Zhang (2013) exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains and release either cellobiose or glucose as major products. Exoglucanases can effectively work on micro-crystalline cellulose, probably break cellulose chains from the microcrystalline structure (Teeri, 1997). Whereas for β-glucosidase that do not contain a catalytic binding modules, it will hydrolyze soluble cellodextrin and cellobiose which act as inhibitor of endoglucanases to glucose (Zhang and Zhang, 2013).

2.5 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae or also known as Baker yeast is a eukaryotic microbe with globular shaped cells as shown in Figure 7, and appear as yellow green yeast, that belongs to the kingdom of fungi (Landry et al., 2006). It has cell walls that are made up of chitin with no peptidoglycan and its lipid is in the form of ester linked biomolecules. Although many microorganisms have been exploited for ethanol production, *S. cerevisiae* still remain as the prime and chosen species for fermentation processes (Bai et al., 2008). According to Legras et al. (2007) there are more than 600 strains of yeast from various geographical origins.

Figure 7: Image of *Saccharomyces cerevisiae* under microscope.
(http://www.microbiologyonline.org.uk/about-microbiology/introducing-microbes/fungi).

One of the important features of *S. cerevisiae* is it abilities to convert sugars into ethanol and carbon dioxide in a short period either during aerobic or anaerobic condition (Hagman & Piskur, 2014). However during ethanol production, yeast can encounter few stress condition either form environment such as nutrient depletion, high temperature and contamination or effect form its own cell metabolism such as ethanol accumulation that can result to the low yield of ethanol as well as reduced the yeast viability (Bai et al., 2008).